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ORIGINAL ARTICLE

Somatic hybrids of *Sinapis alba* + *Brassica juncea*: study of backcross progenies for morphological variations, chromosome constitution and reaction to *Alternaria brassicae*

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Abstract The wild relatives of crops represent a rich reservoir of genes for introducing resilience to climate change into cultivated crops. To introgress genes from *Sinapis alba*, a wild relative of Brassicaceae, into *Brassica juncea*, a cultivated member of this family, we initially produced somatic hybrids between the two species and then produced a large number of backcross populations involving the two somatic hybrids (H1 and H2) with *Brassica juncea*. BC_1 progeny were morphologically very similar. However, when they were challenge inoculated with a highly virulent *Alternaria brassicae* (ITCC No. 2542) culture under in vivo and in vitro conditions in two growing seasons, they showed wide variations in their disease reaction. Of the 40 BC_1 lines tested in one season, 36 showed a resistant reaction. BC_1F_2 progenies derived from these resistant BC_1 plants also showed resistance to *Alternaria brassicae*, indicating stable inheritance of the resistant phenotype. However, BC_1F_2 progenies showed a wide variation in morphological traits, including plant height, basal branching, leaf thickness, trichome density on leaves and stem. BC_1 plants were examined by genomic in-situ hybridization (GISH) to determine their chromosome constitution. All five plants were found to possess 12 strong hybridization signals upon hybridization with a FITC-labeled *S. alba*-specific probe. GISH studies on BC_1F_2 plants indicated localized signals in addition to 12 full chromosome hybridization signals, suggesting alien introgressions into *B. juncea* that requires further validation. The BC_2 generation was found to possess half of the haploid set of alien chromosomes. The BC_1F_2 and BC_2 generations were further screened against *A. Brassiceae* and found to be resistant/tolerant.

Keywords *Sinapis alba* · Genomic in situ hybridization · Alien introgression lines · Genetic variations · Ploidy augmentation · *Alternaria* blight

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Introduction

Brassica juncea (L.) Czern and Coss is a species of mustard plant and a natural allotetraploid oilseed crop grown extensively in more than 53 countries of the world, including India. In the crop year 2016/2017, the area cultivated in *B. juncea* in India accounted for 35% of the world's harvested area of this crop, representing 16% of global production (Darekar and Reddy 2018). However, due to its very limited genetic variability, this oilseed crop is frequently attacked by many diseases, such as Alternaria blight (*Alternaria* spp.), *Sclerotinia* stem rot, white rust and abiotic stresses (drought and high temperature). *Brassica juncea* lacks genetic resistance against *Alternaria brassicae* (Meena et al. 2016), the most prevalent causal agent of blight in oilseed Brassica species. This pathogen severely attacks all areas of mustard cultivation in India and is responsible for up to 47% yield losses (Kolte et al. 1987). However, some wild relatives of Brassica, such as *Camelina sativa*, *Capsella bursa-pastoris*, *Diplotaxis catholica*, *D. erucoides* and *Sinapis alba*, are reported to possess a high degree of genetic resistance against *A. brassicae* (Brun et al. 1988; Conn et al. 1988; Zhu and Spanier 1991; Sharma et al. 2002). Among these wild relatives, *S. alba* has been found to possess a large reservoir of genes conferring resistance to *A. brassicae* (Hansen and Earle 1997; Sharma et al. 2002), *Sclerotinia* stem rot (Li et al. 2009), beet cyst nematode (Lelivelt and Hoogendoorn 1993), flea beetles (Lamb 1984; Brown et al. 2004), pod shattering (Chandler et al. 2005; Wang et al. 2007), high temperature (Downey et al. 1975) and drought (Brown et al. 1997). However, *S. alba* is not readily hybridized with *B. juncea* due to pre- and post-fertilization barriers and, consequently, genome introgression has not been achieved from this wild genus to cultivated Brassica through conventional breeding. Another breeding strategy, sexual hybridization, is commonly used for genome introgression followed by embryo rescue and colchicine-treated genome duplication, but this approach has also had limited success in hybridization attempts between *B. juncea* and *S. alba* (Li et al. 2017). These unsuccessful attempts have led to somatic hybridization being adopted as an alternative approach to construct inter-generic hybrids to augment the ploidy level from allotetraploid to allohexaploid in oilseed Brassicas (Kumari et al. 2018).

Brassicaceae is a model plant family for somatic hybridization, and a large number of attempts have been made to introgress potential genes from alien donor species to crop Brassicas. Unfortunately, these attempts have not succeeded in producing stable and fertile somatic hybrids (Hansen and Earle 1997; Singreva and Earle 1999; Wang et al. 2006). The prevailing sterility in somatic hybrids has been reported to be due to abnormal chromosome pairings, frequent multivalent formation and irregular chromosomal segregation (Gaikwad et al. 1996; Wang et al. 2005b; Sheng et al. 2008), with the result being the failure to produce seeds upon self-pollination. Similar conditions have also been observed in backcross progeny due to the presence of a haploid set of alien chromosomes after the first round of backcrossing (Lelivelt et al. 1993; Begum et al. 1995). This represents a major problem in terms of the stability of allopolyploids. However, a few somatic hybrids have been reported to successfully recover backcross progeny after successive backcrossing (Li et al. 2009; Wang et al. 2013). Therefore, some, albeit limited, information is available on the filial and backcross progeny of allopolyploid Brassica. Although endeavors of previous plant breeders to introgress potential resistant genes from *S. alba* into *B. juncea* have not been successful to date, the gene(s) for yellow seed coat color have been successfully introgressed in *B. napus* from *S. alba* (Wang et al. 2005a). Therefore, to introgress genetic resistance for *A. brassicae* from *S. alba* to *B. juncea* by ploidy augmentation, we have developed the first stable and fertile somatic hybrids of *B. juncea* and *S. alba* which possess a high degree of genetic resistance to *Alternaria* blight disease and high temperature (Kumari et al. 2018).

To introgress invaluable gene(s) into cultivated oilseed Brassicas, we have developed a large number of progenies of the fertile backcross population by using two stable symmetric somatic hybrids (H1 and H2) as a female parent and *B. juncea* cv. RLM-198 and NPJ-212 as a recurrent parent and vice-versa. Regarding these two somatic hybrids, H2 had a recombinant mitochondrial genome and H1 possesses *B. juncea*-type mitochondria while both hybrids acquired chloroplasts from *B. juncea*. The backcross progeny of the hybrids carried resistance to *A. brassicae* due to possessing a haploid and half of the haploid set of *S. alba* after the first and second round of backcrossing, respectively. The agronomic performances of the

second backcross progeny were also found to be promising with a half haploid set of *S. alba*.

The aim of the study reported here was to characterize 53 lines of the 103 backcross progenies for their potential morphological variations, agronomic performances, genomic constitutions with alien introgression and genetic resistance against *A. brassicae* (BC_1F_2 and BC_2) in comparison with earlier reported somatic hybrids.

Materials and methods

The alien introgression lines were derived from two somatic hybrids of *B. juncea* + *S. alba* (H1 and H2) that carried complete chromosomal constitutions (AABBSS, $2n = 60$) of the parent (Kumari et al. 2018). These somatic hybrids were transplanted in the net house of IARI, New Delhi during the 2015–2016 crop season. Both somatic hybrids were used as male and female parents in the first round of backcrossing with *B. juncea* varieties RLM-198 and NPJ-212. Variations in somatic hybrids are reported frequently; therefore, we selected five plants of each hybrid based on morphological variations for backcrossing and selfing simultaneously. The unopened flower buds that were ready to bloom the next day were selected for emasculation, a process carried out with due care to avoid damage to the stigma and bursting of the anthers. The fresh pollens were collected in the early morning from newly opened flowers of *B. juncea* cvs. RLM-198 and NPJ-212 and both somatic hybrids (H1 and H2) and used to pollinate the emasculated buds.

Approximately 45–50 flower buds were backcrossed from each plant to ensure a relatively high seed recovery rate, and at the same time the unpollinated buds were removed to avoid unwanted seed set or selfing. After pollination, the pollinated buds were covered by selfing bags for 5–6 days to avoid outcrossing and pollen contamination. A total of 40 backcross progeny (BC_1) were obtained after the first round of backcrossing, with 25–32 siliques recovered from a single plant and two to four seeds obtained after harvesting from each silique. Approximately 64–80 seeds were obtained from each cross. A total of 30 seeds were sown from each cross in the next crop season at the IARI farm during the 2016–2017 crop season (October–April). Seed germination was very good and calculated to be > 90% in the backcross lines (BC_1). All 40 lines of BC_1 progeny and selfing seeds of the hybrids (H1 and H2) were germinated during the 2016–2017 crop season. Of these 40 BC_1 lines, four were found to be *B. juncea* type in terms of their morphology and resistance responses; these were not used in subsequent crosses. All of the remaining 36 true BC_1 lines obtained after backcrossing were used for the development of the BC_2 generation with their respective parents (*B. juncea* cvs. RLM-198 and NPJ-212) and reciprocal crosses made in all lines, with this second round of backcrossing producing 72 BC_2 lines. At the same time, all 36 backcrossed lines (BC_1) were selfed to produce BC_1F_2 seeds. All BC_1F_2 and BC_2 lines were used for morphological characterization and resistance screening in the 2018 offseason period at Katrain. The flow diagram shown in Fig. 1 illustrates the procedure used to develop backcross progenies from somatic hybrids.

Morphological characterizations of backcross progenies

The backcross progeny obtained after successful hybridization of somatic hybrids and *B. juncea* were grown in the agriculture field of the Indian Agriculture Research Institute, New Delhi. Of 103 lines, 53 backcross lines (36 BC_1F_2 and 17 BC_2 lines) showing highly variable morphological characteristics were selected for morphological study, and the important morphological characters that were found variable between the populations were recorded. The features studied included plant height, number of primary and secondary branches, length of the main shoot, days after sowing to flowering, the total number of pods on the main shoot, length of silique, length of the beak and number of seeds per silique at maturity. Seed coat

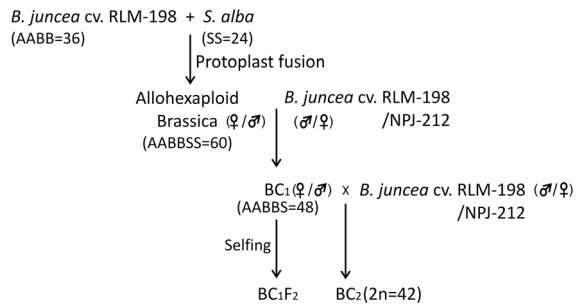

Fig. 1 Flow chart of the development of backcross (*BC*) progenies from somatic hybrids

color was also studied in the backcross population, somatic hybrid and parents.

Chromosome preparation and genomic in situ hybridization

To prepare slides for the mitotic studies, we first sowed ten seeds of each BC_1F_2 ($n = 36$) and BC_2 ($n = 17$) line on moist filter papers in Petri dishes at room temperature. Following germination, the roots (length 15 mm) of 2-day-old seedlings were sliced and pretreated with hydroxyquenoline for 3 h, then immersed in cold water for 2 h, followed by fixation in Carnoy's solution (alcohol: glacial acetic acid [3:1]) overnight, after which they were transferred into 70% ethanol for storage until needed. For examination, the root preparations were first treated with a mixture of 2% cellulase (v/v; Sigma, St. Louis, MO, USA) and 2% pectinase (v/v; Sigma) for 1 h at room temperature; then gentle pressure was applied to squeeze out the mitotic cells, which were stained with one drop of 2% acetocarmine and covered with a coverslip. The slides were then warmed directly over a burner and the cells dispersed and cleaned with 45% acetic acid. Finally, the prepared slides were dipped in liquid nitrogen and the coverslip was flicked off before tget were stored in absolute alcohol.

The genomic DNA of *B. juncea* and *S. alba* was isolated using the CTAB method (Kirti et al. 1995) and purified. The sheared *S. alba* genomic DNA was labeled with fluorescein-12-dUTP using a nick translation kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. Genomic in situ hybridization (GISH) was performed using 30 µl of hybridization mixture (50% formamide, 10% 20× SSC, 20% dextran sulphate, 200 ng of labeled DNA of *S. alba* probe and 100-fold excess of sheared *B. juncea* genomic DNA applied as a blocking DNA) as follows. The slide was incubated at 80 °C for 2 min in a thermocycler for denaturation, then 30 µl of hybridization mixture was added and the slide covered with plastic coverslip. These slides were then incubated overnight at 37 °C in a moist chamber for hybridization. Post-hybridization washing consisted of three 5-min washes in 2× SSC, one 10-min wash in 50% formamide in 2× SSC and two 5-min washes in 2× SSC, all at 42 °C in a waterbath, followed by one 5-min washing in 2× SSC at room temperature. The slides were counterstained with 2 mm DAPI and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA). The slides were visualized by fluorescence microscopy (Imager Z2 AX10 microscope; Carl Zeiss Microscopy GmbH, Jena, Germany).

Resistance screening for *A. brassicae*

The virulent *A. brassicae* culture was procured from ITCC, IARI, New Delhi (ITCC No. 2542) and maintained on Brassica dextrose agar medium at 4 °C for use in artificial inoculation of the backcross progenies. After 96 h, the conidia were harvested from the culture into sterilized double-distilled water. The conidial concentration was maintained at 10^6 ml^{-1} in ddH_2O. The lower leaves of the backcross progenies (BC_1F_2 and BC_2) were inoculated with a pathogen spore suspension and by sticking conidial discs onto the leaves. The humidity was maintained by spraying the plants with sterilized distilled water for 7 successive days. The blight lesion size (lesion size = length × width) was recorded from days 7 to 10 after the inoculation of the pathogen. The resistance or susceptibility of an individual backcross line, i.e. the resistance response, was estimated according to the percentage blighted leaf area (BLA) as: no lesion (immune); 0–10% BLA (highly resistant); 11–20% BLA (resistant); 21–30% BLA (moderately resistant); 31–40% BLA (tolerant); 41–50% BLA (moderately tolerant); 51–60% BLA (susceptible); and > 60% BLA (highly susceptible). The complete screening experiment was conducted during 2017 (rabi season) at IARI, New Delhi and 2018 (offseason) at the IARI-Regional Station, Katrain (Himachal Pradesh).

Results

All of the BC_1 progenies had an almost uniform morphology and possessed a high degree resistance to *A. brassicae*. The 36 BC_1F_2 lines and 72 BC_2 lines were sown in the 2018 crop and offseason period for assessment of morphological characters and resistance screening against *A. brassicae*. All 36 BC_1F_2 lines but only 67 BC_2 lines germinated; of these, all 36 BC_1F_2 lines showed remarkable variability in morphological parameters and thus were selected for further study while only 17 of the 67 BC_2 lines showed significant variability at the morphological level and selected for

further study. Those siblings of the BC_2 lines that did not show significant variability at the morphological level were not studied further. Consequently, of the 103 backcross lines developed for the study, only the 53 (36 BC_1F_2 and 17 BC_2) lines showing significant variability in morphological parameters were studied in detail, also for chromosomal status.

Morphological variations in backcross progenies

The 53 BC_1F_2 and BC_2 lines chosen for further study showed variability for morphological characteristics when compared to the somatic hybrids and the parents (*B. juncea* and *S. alba*). All plants of the BC_1F_2 lines grew vigorously and were taller than the parents; in comparison, plants of the BC_2 lines did not grow as vigorously as those of the BC_1F_2 lines and plant height was also decreased in some lines (Fig. 2f, g). Plant height was surprisingly variable among the 36 BC_1F_2 lines, with the maximum height recorded for plants of line 11 (308.33 cm) (Fig. 3e, f), and the smallest plants, with an average height of 156.67 cm, observed for line 1. However, the majority of BC_1F_2 lines attained a plant height of > 200 cm. In comparison, plants of the BC_2 lines attained a maximum height of 240 cm (Fig. 3d) and a minimum height of 169 cm (lines 44 and 48, respectively). The maximum number of primary branches (22.75) and secondary branches (116) were recorded in the plants of lines 44 and 51, respectively. There were considerably more basal branches in plants of the BC_1F_2 lines than in plants of the second round of backcross progenies. All plants of the BC_1F_2 lines had more primary branches than the parents, which resulted in BC_1F_2 plants having a

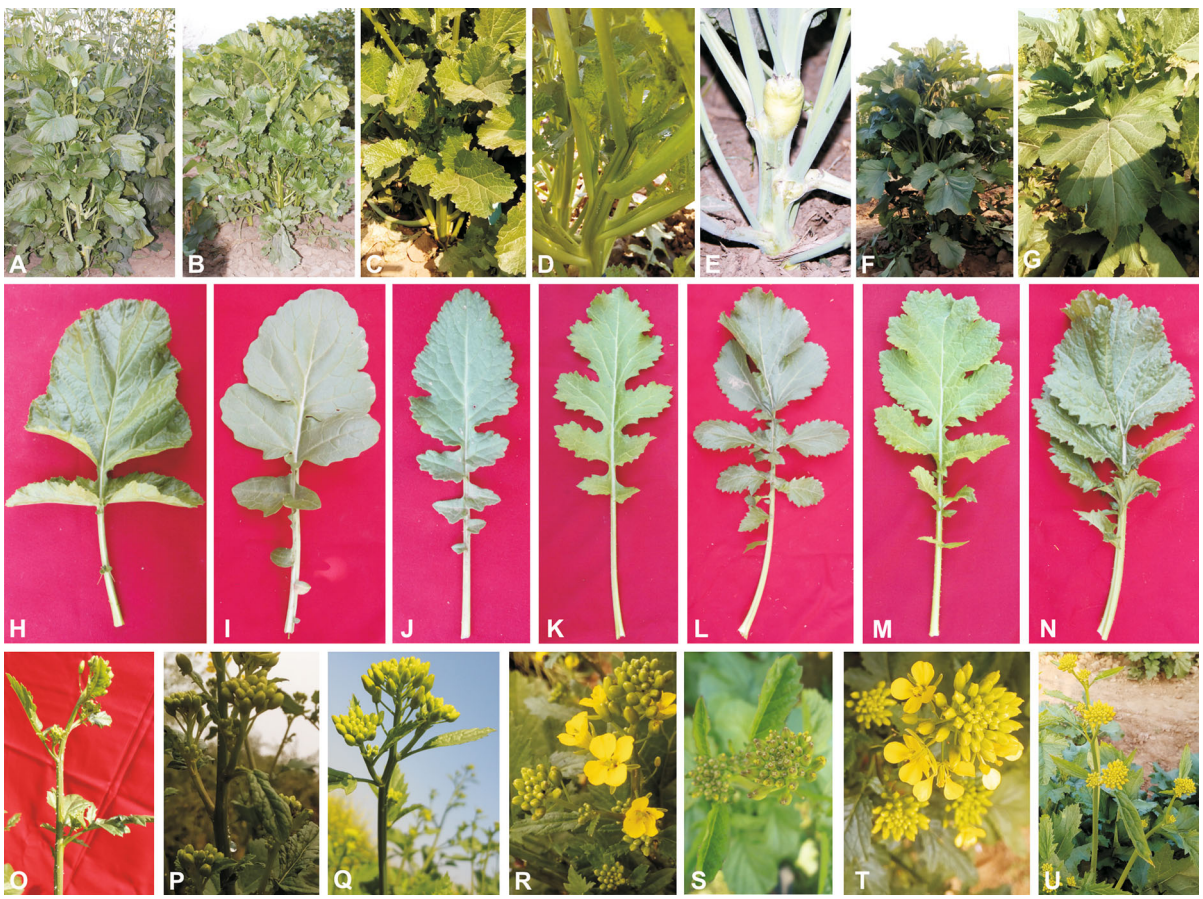

Fig. 2 Morphological variations in the BC_1F_2 and BC_2 progenies of *Brassica juncea* + *Sinapis alba* somatic hybrids. **a–e** Morphology of BC_1F_2 progenies (**a–c**), with deep grooved rough stem (**d**) and knot-like structure on stem (**e**). **f**, **g** Morphology of BC_2 plants. **h–n** Variations in leaf shapes and margins in BC_1F_2 (**h–l**) and BC_2 (**m**, **n**) lines. **o–u** Flower buds of different color, shape and size from BC_1F_2 (**o–s**) and BC_2 (**t**, **u**) lines

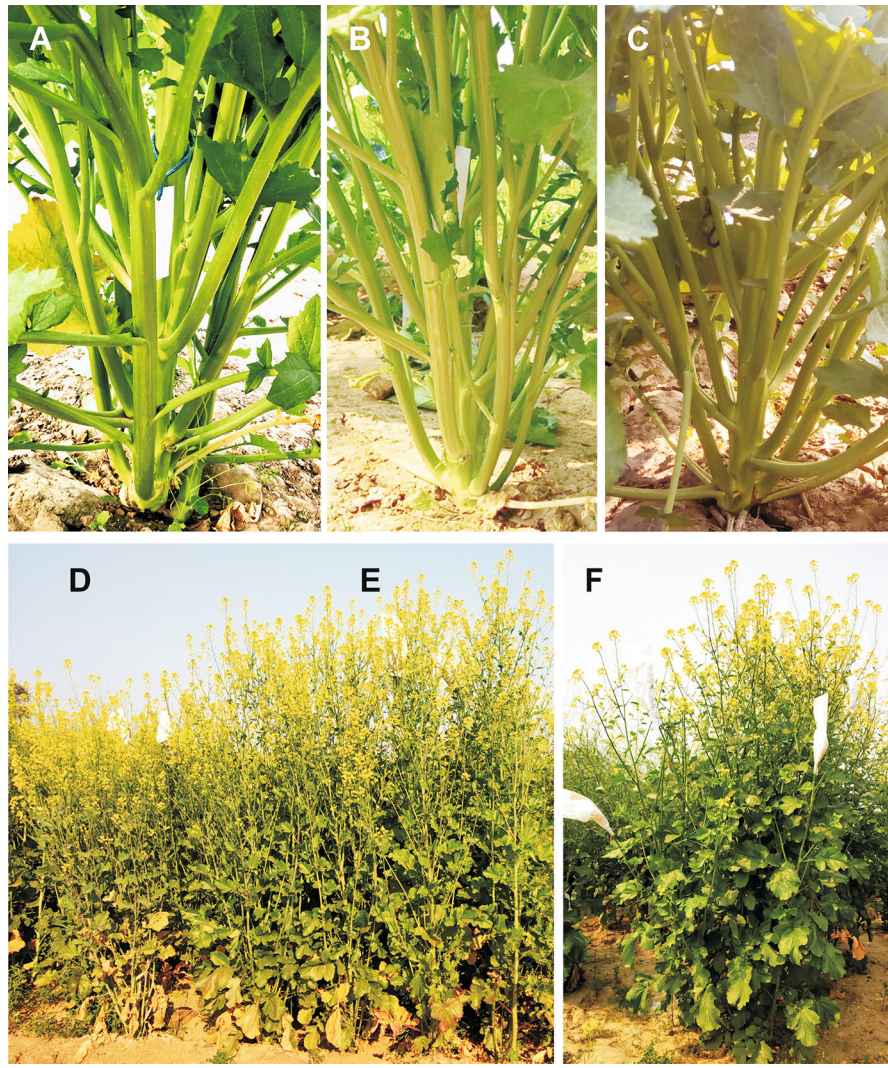

Fig. 3 Backcross progenies (introgression lines) showing interesting phenotypes. **a–c** Branching patterns in BC_1F_2 lines, **d–f** height of BC_2 (**d**) and BC_1F_2 (**e**, **f**) progenies

unique bushy appearance (Fig. 2a–c). Of all the lines analyzed, plants of line 33 produced the highest number of primary branches (27.66) followed by those of line 16 (26.67) (Fig. 3a–c), and plants of line 4 produced the lowest number of primary branches (11.33). Plants of line 22 produced the maximum number of secondary branches (166.67) of all plants analyzed, and those of line 9 produced the lowest number (29.67).

Two lines of BC_1F_2 progeny developed a hard knot-like structure on the main stem at about 15 cm above the soil line which gave the plant a cabbage head-like appearance (Fig. 2e). All lines of both backcross progenies showed recognizable variations in leaf shapes and sizes. The shape of the leaf blades after two rounds of selfing of backcross progenies was ovate to lyrate, with the majority having dentate margins but undulating margins also appeared. The leaves had a highly dissected trifoliate to multi-foliate lyrate structure, were deeply lobed (1–3 lobes), thick and leathery and had a deep-green color (Fig. 2h–l). However, highly modified leaves were observed in the BC_1F_2 progenies which had very fine wire-like persistent tendrillar stipules on the petiole, which are not normally present in family Brassicaceae (line 23) (Fig. 2h). The leaf blade possessed prominent

trichomes on the upper and lower surface (like a somatic hybrid), specifically along the margins, while some leaves had acquired very few trichomes. The BC_2 progenies were covered with dense leaves, and their leaves possessed fewer trichomes compared to plants of the BC_1F_2 lines (Fig. 2m, n). The surface of the stems was rough with deep grooves bearing dense or sparingly present trichomes (Fig. 2d). The color of the stems was highly variable, with some lines having a purple color at the initiation point of the primary and secondary branches while some stems were completely purplish-green (BC_1F_2 lines 12, 15, 23, 24, 35); however, stems of other lines were completely green. Three BC_1F_2 progenies secreted a transparent sticky gum-like exude from the stem surface under drought and heat stress conditions (lines 4, 32, 34) (Fig. 2o, p).

The inflorescence was highly variable between both generations of backcross progenies. The BC_1F_2 plants had compact buds, while the BC_2 plants had a whorled shape and spaced inflorescences. In BC_1F_2 plants, the flower buds were approximately circular (lines 7, 10, 17, 20) (Fig. 2q, r), and in some plants they were purple at the upper tip with white linings at the outer side of the calyx. The stigma emerged first from unopened flower buds in these plants (lines 15, 16, 19) (Fig. 2s). In comparison, the BC_2 lines bore many identical buds to *B. juncea* in terms of shape, size and anthesis (i.e. completely green or light greenish-yellow buds that were approximately rectangular in shape (Fig. 2t–u).

The length of the main shoot was longer in BC_2 progenies than in BC_1F_2 plants. Recognizable variations were noted in silique size and the beak between hybrids and the BC_1F_2 and BC_2 progenies. The silique in the BC_2 progenies was appreciably longer than that in the hybrid. The BC_1F_2 progenies had the highest average silique length (4.92 cm), but BC_2 progenies had larger siliques (7.02 cm) in line 34 than the hybrid (3.16 cm) and cultivated parent B. juncea (5.41 cm) (Fig. 4d). Similarly, the average number of seeds per silique also increased from the hybrids to the BC_2 progenies. BC_2 line 38 had more seeds per silique (19.86) than *B. juncea* (11.90). The average minimum number of seeds per pod was recorded in BC_1F_2 line 36 (5.5) (Table 1). However, beak size was noted to be larger in most of the BC_1F_2 lines as compared to the BC_2 progeny and recurrent parent *B. juncea*. In BC_1F_2 lines, the maximum beak length was recorded in lines 9 and 11 (1.16 cm in both lines), and the shortest beak was found in BC_2 lines 37 and 48 (0.7 cm). The seed coat color of *B. juncea* was dark brown/black and *S. alba* has yellow seeds. The somatic hybrids produced dark-brown seeds. The seed coat color of plants of the BC_1F_2 and BC_2 generations varied from that of the recurrent parents and somatic hybrids in being different shades of brown. Seed size was also increased in the BC_2 generation, with bold-sized seeds harvested in the majority of lines (data not shown).

GISH analyses of backcross progenies

The backcross progenies that differed morphologically were selected for the cytological studies. A total of 21 lines were selected for the GISH analysis, of which four were BC_1 lines, ten were BC_1F_2 lines and seven were BC_2 lines. The somatic cells of all lines analyzed had the expected chromosome numbers. The presence of alien chromosomes was confirmed by using FITC-labeled *S. alba* as a probe, with *B. juncea* and *S. alba* chromosomes stained red and green, respectively. As expected, the mitotic studies of BC_1 plants showed a complete haploid set (S, $n = 12$) of *S. alba* chromosomes (green) together with the diploid set (AABB, $2n = 36$) of *B. juncea* chromosomes (red) (Fig. 4a). We reported previously that meiotic studies showing normal bivalent pairing and separation of chromosomes maintained complete pollen and pistil fertility (Kumari et al. 2018). The mitotic studies of the BC_1F_2 progenies indicated localized signals in addition to the presence of 12 full chromosome hybridization signals, suggesting alien segmental introgression into *B. juncea* (Fig. 4b). However, further validation of introgression is required in the selfing progenies of backcrossed population of somatic hybrids. Similarly, in our mitotic studies of the BC_2 generation revealed the presence of six chromosomes (Fig. 4c) together with a diploid set of *B. juncea*. We counted 42 chromosomes in mitotic studies of BC_2 generation. We expect that the BC_2F_2 progenies will also be subject to some recombination.

Resistance screening to *A. brassicae*

Disease screening was performed in two consecutive seasons at two different locations, and the data are reported as the mean of both seasons. Fourteen lines in BC_1F_2 generation produced dark-green, thick and leathery leaves; these lines were recorded as being

Table 1 Morphology and variations in response to *Alternaria* blight disease of backcrossed progenies (BC_1F_2 and BC_2) of *Brassica juncea* + *Sinapis alba* somatic hybrids

Backcrossed progenies	*Alternaria* blight response[a]	No. of siliques on main shoot	Main shoot length (cm)	Silique length (cm)	Silique beak length (cm)	No .of seeds/ silique	Plant height (cm)	No. of primary branches	No. of secondary branches
BC_1F_2 line no.									
1	MR	32.33 ± 6.23	51.33 ± 11.14	3.97 ± 0.26	0.79± 0.1	12.34 ± 2.86	156.67 ± 4.99	15 ± 3.74	47.33 ± 9.8
2	R	41.33 ± 7.54	50.33 ± 8.95	3.51 ± 0.34	0.86± 0.13	12.9 ± 4.06	198 ± 11.56	16.33 ± 2.49	76 ± 12.32
3	R	36 ± 4.89	41.33 ± 6.01	4.47 ± 0.43	0.84 ± 0.12	14.68 ± 1.53	176.33 ± 4.18	12.67 ± 2.86	48.33 ± 14.19
4	R	59 ± 8.6	65.83 ± 11.43	4.76 ± 0.21	0.89 ± 0.1	15.21 ± 2.22	207.67 ± 17.24	11.33 ± 1.7	48.33 ± 2.35
5	R	47.33 ± 9.56	65.47 ± 10.18	4.92 ± 0.2	0.85 ± 0.16	14.78 ± 1.95	182.67 ± 24.56	13.33 ± 1.33	60 ± 10.57
6	R	63.33 ± 13.42	49.17 ± 12.62	4.45 ± 0.57	0.97 ± 0.3	14.6 ± 0.45	225.67 ± 14.52	15 ± 0.8	43 ± 2.44
7	MR	46.25 ± 8.84	52.87 ± 14.77	3.23 ± 0.52	0.76± 0.11	8.78 ± 3.83	194.75 ± 10.49	16.5 ± 2.17	59.5 ± 18.72
8	R	50 ± 4.4	61.25 ± 3.26	4.26 ± 0.36	0.82 ± 0.12	14.37 ± 2.21	228.25 ± 2.27	14.5 ± 6.65	45.25 ± 20.5
9	HR	37.67 ± 11.81	32.67 ± 8.95	3.47 ± 0.54	1.16 ± 0.25	8.96 ± 3.26	232 ± 2.44	15.33 ± 1.24	29.67 ± 1.69
10	HR	49 ± 4.96	57 ± 4.96	3.98 ± 0.75	0.92 ± 0.15	10.65 ± 4.35	234.33 ± 1.69	16.33 ± 1.24	56 ± 7.87
11	HR	45.33 ± 9.97	40 ± 10.8	3.04 ± 0.27	1.16 ± 0.18	6.4 ± 2.21	308.33 ± 9.39	17.67 ± 1.24	48.67 ± 8.17
12	HR	54.8 ± 11.01	44 ± 8.12	3.06 ± 0.36	1.12 ± 0.2	6.68 ± 1.69	257 ± 33.75	17.6 ±1.85	52 ± 6.1
13	HR	47.25 ± 6.61	41.25 ± 4.32	3.35 ± 0.32	1.2 ± 0.2	7.71 ± 1.62	289.25 ± 10.42	25.5 ± 3.35	78 ± 15
14	HR	51 ± 4.3	43.25 ± 5.8	3.26 ± 0.33	1.14 ± 0.16	7.27 ± 1.96	284.75 ± 11.45	22.75 ± 2.58	55.25 ± 15.94
15	HR	45.67 ± 1.24	47.33 ± 7.04	2.99 ± 0.42	1.1 ± 0.21	7.51 ± 1.81	245 ± 8.52	23 ± 4.08	74.67 ± 33.67
16	R	45 ± 5.88	37.67 ± 7.93	3.2 ± 0.34	1.07 ± 0.19	7.33 ± 1.59	289.67 ± 19.68	26.67 ± 2.62	62.67 ± 3.3
17	HR	48.33 ± 5.9	57 ± 3.74	2.99 ± 0.38	0.99 ± 0.16	6.06 ± 1.45	249 ± 8.98	21.67 ± 1.24	87.67 ± 23.01
18	R	46.33 ± 4.71	43.67 ± 5.8	2.94 ± 0.43	0.92 ± 0.16	8.17 ± 2.22	213.33 ± 2.86	18 ± 2.94	57 ± 6.97
19	R	49.33 ± 5.69	47.67 ± 11.24	3.32 ± 0.4	0.91 ± 0.24	9.3 ± 1.8	181.33 ± 6.11	16.33 ± 2.5	46.67 ± 6.4
20	HR	41 ± 10.34	51.25 ± 16.57	3.88 ± 0.45	1.1 ± 0.25	9.68 ± 1.97	202.67 ± 16.81	16.5 ± 3.7	73.5 ± 12.35
21	R	40.33 ± 7.14	31.67 ± 3.04	2.81 ± 0.31	0.96 ± 0.21	7.27 ± 1.5	224 ± 36.11	20 ± 5.71	56 ± 17.14
22	HR	48.33 ± 3.13	42 ± 3.56	2.93 ± 0.34	1.05 ± 0.2	7.53 ± 1.28	267.67 ± 18.6	24.67 ± 3.93	166.67 ± 25.2
23	HR	57 ± 3	30 ± 0.0	2.97 ± 0.3	1.18 ± 0.1	6.8 ± 1.4	210.5 ± 10.5	19.5 ± 0.5	80.5 ± 13.5
24	R	42.33 ± 10.2	47.5 ± 1.78	3.55 ± 0.34	0.85 ± 0.12	10.03 ± 3.08	225.33 ± 3.85	15 ± 2.9	46.33 ± 19.13
25	R	32.33 ± 3.3	33.67 ± 5.8	3.88 ± 0.66	0.91 ± 0.18	14.2 ± 2.05	208.33 ± 15.17	16.67 ± 2.05	59.67 ± 8.3
26	R	38.33 ± 1.24	40.33 ± 4.4	3.7 ± 0.82	0.93 ± 0.11	10.59 ± 5	226.33 ± 2.6	20.66 ± 1.2	77.67 ± 9.7
27	R	39.67 ± 6.1	54.33 ± 8.9	4.76 ± 0.57	0.92 ± 0.1	13 ± 3.25	204 ± 9.9	18 ± 0.81	73.33 ± 10.6
28	MR	48 ± 0.81	48.33 ± 1.2	4.14 ± 0.45	0.83 ± 0.14	15.03 ± 1.66	222.33 ± 1.7	20.33 ± 1.7	50.67 ± 3.09
29	R	43.67 ± 2.05	60.67 ± 3.3	4.8 ± 0.7	1.05 ± 0.2	15.07 ±1.4	228 ± 3.2	19.66 ± 1.2	69 ± 8.8
30	MR	42 ± 17.7	61.83 ± 19.7	4.77 ± 0.24	0.81± 0.9	15.61 ± 1.76	227.33 ± 6.9	15.66 ± 1.7	84.33 ± 39.96

Table 1 continued

Backcrossed progenies	*Alternaria* blight response[a]	No. of siliques on main shoot	Main shoot length (cm)	Silique length (cm)	Silique beak length (cm)	No .of seeds/ silique	Plant height (cm)	No. of primary branches	No. of secondary branches
31	HR	45.5 ± 4.56	36.25 ± 3.85	3.06 ± 0.36	1 ± 0.15	7.24 ± 1.14	292 ± 1.94	26.5 ± 3.36	67.5 ± 14.18
32	HR	63.5 ± 12.3	44.25 ± 4.09	2.89 ± 0.45	1.04 ± 0.17	5.86 ± 1	244 ± 21.12	21.5 ± 1.7	71.5 ± 13
33	R	49 ± 4.56	51.33 ± 9.8	3 ± 0.33	0.97 ± 0.2	7.52 ± 1.5	248.67 ± 3.5	27.66 ± 2	142.33 ± 11.9
34	HR	45.25 ± 9.5	45.25 ± 10	3.13 ± 0.28	1.09 ± 0.22	7.72 ± 1.5	251.15 ± 5.5	14 ± 3.6	59.25 ± 9.4
35	HR	36.75 ± 5.7	38.75 ± 5.3	3.05 ± 0.4	1.16 ± 1	6.32 ± 2.5	253.9 ± 12.5	24.5 ± 2.1	108.75 ± 25.65
36	R	45.5 ± 0.70	46 ± 1.4	2.93 ± 0.5	0.92 ± 0.22	5.5 ± 1.7	174 ± 8.4	14.5 ± 0.7	84 ± 16.9
BC_2 line no.									
37	T	51.67 ± 4.49	47 ± 10.8	5.8 ± 0.68	0.7 ± 0.19	17.5 ± 4.7	182 ± 9.4	19.33 ± 8.4	69.33 ± 40.66
38	MR	49 ± 7.34	65.67 ± 13.47	7.02 ± 0.21	0.8 ± 0.1	19.86 ± 2.07	199 ± 4.96	14.33 ± 0.94	50 ± 13.06
39	R	59.67 ± 7.03	69.67 ± 10.33	6.21 ± 0.41	0.77 ± 0.14	18.08 ± 2.85	225.67 ± 16.21	16 ± 0.81	53.33 ± 11.46
40	T	56.67 ± 2.86	58.23 ± 1.35	6.28 ± 0.17	0.85 ± 0.09	18.06 ± 0.92	218 ± 2.16	14.67 ± 4.1	53.33 ± 4.1
41	MR	54 ± 4.24	59.9 ± 8.48	5.98 ± 0.99	0.83 ± 0.18	16.28 ± 5.97	227.67 ± 3.3	15.67 ± 1.24	55.67 ± 3.68
42	R	57.67 ± 10.07	70.83 ± 1.43	6.55 ± 0.2	0.8 ± 0.09	18.9 ± 1.53	234.67 ± 4.1	12.33 ± 0.94	55.67 ± 21.35
43	R	52.67 ± 2.05	66.5 ± 12.78	6.34 ± 0.31	0.78 ± 0.11	16.28 ± 1.28	231.67 ± 3.68	12.67 ± 1.24	34.67 ± 4.92
44	T	43 ± 6.58	47.25 ± 8.61	5.33 ± 0.72	0.85 ± 0.12	16.1 ± 4.54	240.25 ± 11.05	22.75 ± 3.3	109.5 ± 9.12
45	T	63.33 ± 3.51	61.33 ± 11.08	6.09 ± 0.18	0.84 ± 0.1	17.59 ± 1.37	209 ± 4.29	16.33 ± 1.23	72 ± 12.32
46	T	52 ± 6.64	68.33 ± 7.36	6.6 ± 0.28	0.84 ± 0.13	17.41 ± 2	212.33 ± 3.04	20 ± 1.8	100 ± 32.89
47	MR	38.33 ± 2.08	49 ± 9.12	6 ± 0.3	0.81 ± 0.16	16.3 ± 1.5	207 ± 18.9	13.67 ± 3.5	53 ± 18.9
48	R	39.67 ± 2.8	42.66 ± 2.5	5.69 ± 0.2	0.7 ± 0.12	16.09 ± 2.52	169 ± 20.2	13 ± 0.81	49 ± 8.28
49	R	51.67 ± 6.12	54 ± 1.41	6.31 ± 0.35	0.77± 0.11	16.18 ± 1.4	233 ± 3.5	15.33 ± 3.8	55.67 ± 19.5
50	T	51.33 ± 7.3	56 ± 2.9	6.3 ± 0.3	0.98 ± 0.2	16.17 ± 2.6	225.33 ± 4.9	19.33 ± 1.2	76.67 ± 4.9
51	MR	58.66 ± 4.02	61 ± 5.7	6.77 ± 0.49	0.8 ± 0.18	16.6 ± 1.7	222 ± 10.7	21.33 ± 2.05	116 ± 30.53
52	R	54 ± 9	59.37 ± 10.2	6.84 ± 0.2	0.73 ± 0.08	17.24 ± 2.6	207.75 ± 8.7	15.33 ± 2.2	24.5 ± 3.4
53	R	60.66 ± 2.5	62.25 ± 9	6.3 ± 0.18	0.72 ± 0.1	16.1 ± 1.8	222.67 ± 2.3	20.33 ± 2.3	70.67 ± 18.3

Values are presented as the average ± standard deviation

[a]Measured as pecentage blighted leaf area (BLA): HR, highly resistant (0–10% BLA); R, resistant (11–20% BLA); MR, moderately resistance (21–30% BLA); T; tolerant (31–40% BLA)

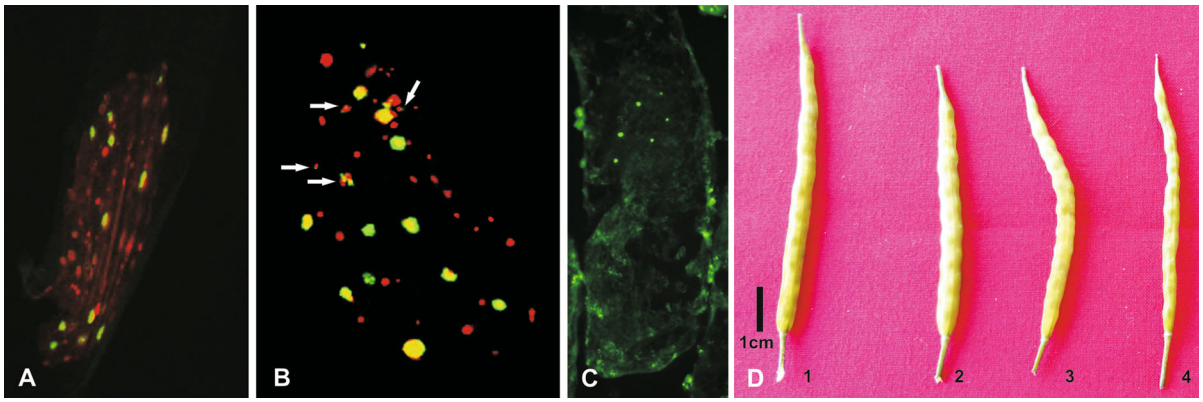

Fig. 4 Genomic in situ hybridization of BC_1, BC_1F_2 and BC_2 generations. **a** BC_1 progeny showing *S. alba* (green) and *B. juncea* (red) chromosomes without introgression, **b** segmental introgression of *S. alba* genome in *B. juncea* chromosomes (arrow) in BC_1F_2 progeny, **c** *S. alba* chromosomes in BC_2 progeny, **d** silique size in BC_2 generation

highly resistant to *A. brassicae* as the virulent pathogen was unable to grow on the leaves and develop typical disease lesions upon inoculation. These leaves showed a hypersensitive reaction against the pathogen upon pathogen challenge, with the inoculated leaf parts drying up and falling off to prevent further extension of the pathogen (Fig. 5e). Eighteen BC_1F_2 lines developed disease lesions of the pathogen, but these were few (1–4) and small-sized; thus, these lines were categorized as resistant (Fig. 5a–h). The light-pigmented leaves with thin lamina produced smaller-sized lesions and these lines were categorized as showing moderate resistance to the disease (lines 1, 7, 28, 30). A thick leaf with a

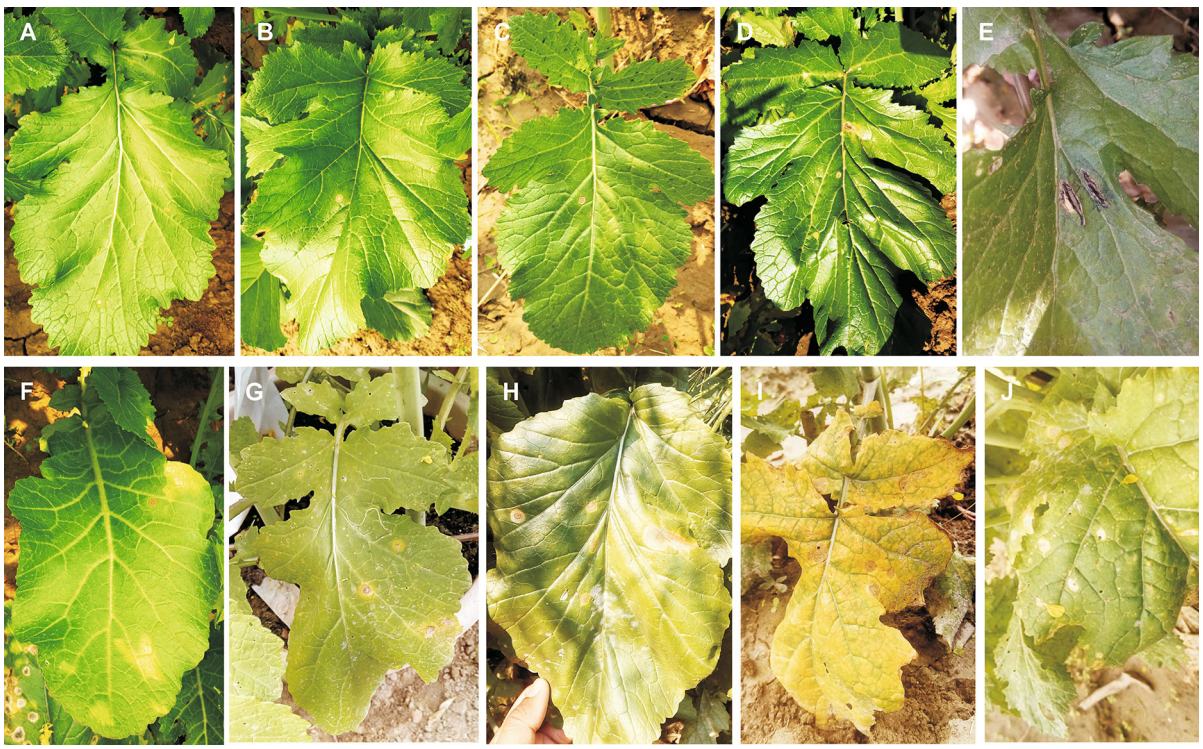

Fig. 5 Screening for *Alternaria* blight disease responses in BC_1F_2 and BC_2 progenies. **a–h** Resistance reactions BC_1F_2 lines, **i, j** moderately resistance responses of BC_2 lines

leathery texture and deep-green color was observed to be directly correlated with resistance responses against the *A. brassicae* pathogen. Similarly, the virulent strain did not survive on plants that had the characteristic beak shape of *S. alba*; thus, these plants were identified as showing resistance to the disease. The incidence of disease incidence was higher in plants of the BC_2 generation, with seven introgression lines found to be resistant to the disease developing blighted lesions on leaves following challenge by *A. brassicae* (lines 39, 42, 43, 48, 49, 52, 53). Four lines (38, 41, 47 and 51) were found to be moderately resistant (Fig. 5i, j) and six lines (37, 40, 44, 45, 46, 50) were found to be tolerant for the disease (Table 1). The progenies recovered after the first round of backcrossing and consecutive selfing (BC_1F_2) were found to be significantly more resistant than the BC_2 plants and their susceptible parent *B. juncea*. None of the BC_1F_2 lines were found to be susceptible to the disease.

Discussion

In the present study, we report our study of backcross progenies derived from two somatic hybrids of *S. alba* + *B. juncea* that carry genetic resistance to *A. brassicae*. These backcross progenies showed variation in morphological characteristics and in their level of resistance to *A. brassicae*. To our knowledge, the is the first report of very good male and female fertility in backcrossed progenies of somatic hybrids, although infertility in backcross progenies of somatic hybrids has been reported earlier (Lelivelt et al. 1993; Singreva and Earle 1999). Therefore, there is a need to maintain fertility in *Alternaria* blight-resistant introgression lines in order to transfer genetic resistance into other cultivated Brassicas and to identify the genomic regions governing resistance for *Alternaria* blight disease. The *Alternaria* blight resistance gene is highly sought by Brassica breeders, but unfortunately to date all efforts have been unsuccessful due to the unavailability of a plant population showing differential resistance expression combined with high fertility. However, many attempts have been made to introgress resistance from wild relatives into cultivated crops. Gaikwad et al. (1996) failed to introgress resistance from *S. alba* to *B. juncea* due to the appearance of male sterility in the somatic hybrids. These hybrids lost their fertility due to multivalent formation and abnormal segregation of the meiotic chromosomes. Begum et al. (1995) produced somatic hybrids of *B. juncea* and *D. harra* but did not succeed in recovering filial generation. These hybrids produced completely infertile pollens due to irregular separation of meiotic chromosomes at anaphase II. Similarly, Hansen and Earle (1997) failed to transfer *Alternaria* blight resistance from *S. alba* to *B. oleracea* due to the production of nonviable pollens. Thus, genome instability has been a common problem throughout studies in inter-generic somatic hybrids, their filials and backcross progenies. Nonetheless, our group has been able to develop not only stable and fertile somatic hybrids but also their fertile backcrossed progenies (Kumari et al. 2018). Wang et al. (2005a) were unsuccessful in producing fertile somatic hybrids but they did recover the backcrossed generation of a somatic hybrid of *B. napus* and *S. alba* and successfully introgressed yellow seed coat colur.

The novel achievement of this study is that all lines of the backcross progenies possesses a high degree of male and female fertility. Surprisingly, we have not yet found any male or female sterile plant in the backcrosses and consecutive selfing generations. However, the backcross progenies did vary morphologically in terms of plant height, leaf shape and size and silique size and in resistance responses. The leaves were ovate to lyrate in shape with undulating and dentate margins. The stems varied from being smooth to being deep grooved, with either dense or sparsely dispersed trichomes. Nothnagel et al. (1997) also reported morphological variations in the backcross progeny of *B. oleracea* and *S. alba* somatic hybrids. The size of the silique in the BC_2 lines were twofold larger than those in the somatic hybrid and *B. juncea* parent and there was an increased number of seeds per silique. Li et al. (2009) found appreciable enlargement in silique size from the BC_1 to $BC1F_4$ generation.

Our mitotic studies of the backcross progeny using GISH suggested the presence of a complete haploid set of *S. alba* in plants of the BC_1F_2 generation with possible segmental introgressions of *S. alba* within *B. juncea* chromosomes, this observation needs further validation. In an earlier study, we found proper pairing and separation during meiosis in BC_1 progeny (Kumari et al. 2018). This pairing could be due to genomic similarities between *S. alba* and *B. juncea* because both genomes share the same 'Nigra' lineage of

subtribe Brassicinae (Warwick and Black 1991; Nelson and Lydiate 2006). We also found about 78% hit contigs in the *S. alba* genome on local BLASTN with *B. juncea*, which confirmed a high genetic similarity between both genera (Kumari et al., unpublished results). The high homology between the *S. alba* and *B. juncea* genomes could be responsible for normal chromosomal pairing and segregation. The segmental introgressions of *S. alba* into *B. juncea* chromosomes might have created morphological variations in the BC_1F_2 progenies; this possibility requires further study. Wang et al. (2005b) obtained the expected mitotic chromosome constitution in the BC_1 generation of *B. napus* and *S. alba* somatic hybrids. However, these researchers found univalent and trivalent formations during meiosis in the backcrossed plants and aneuploidy, which were not observed in our studies.

The resistance to *A. brassicae* was found to be correlated with the shape and texture of the leaves. The pathogen failed to thrive on BC_1F_2 lines bearing thick and leathery leaves, with the plants showing hypersensitive reactions upon challenge with highly virulent strain under favorable field and in vitro conditions. The thickness of the stem was also found to be correlated with resistance to *Sclerotinia* stem rot (Li et al. 2006). Similarly, the shape of the silique and beak size were found to be correlated with the resistance nature of backcross progenies for *Alternaria* blight disease. We found that those lines which produced *S. alba*-like siliques with a characteristic beak had a high degree of resistance to the disease. Hansen and Earle (1995) recovered backcross progeny (BC_1) with the recurrent parent *B. napus* (resistant to black rot) from somatic hybrids of *B. oleracea* and *B. napus*. These researchers reported the same resistance level in the BC_1 generation against *X. campestris* pv. *campestris* as was present in somatic hybrids; however, the BC_1 plants showed different morphological characteristics from both parents and among each other. Similar morphological variation was reported by Li et al. (2009) in backcross progenies derived from *B. napus* and *S. alba* somatic hybrids. These researchers reported different silique sizes in the backcross progenies and have introgressed many agronomically important traits into *B. napus*, including resistance to *Sclerotinia* stem rot.

In the present study, all backcross lines were found to be easily crossable with other cultivated diploid and allotetraploid Brassicas. Therefore, these lines of the backcross progenies are novel genetic resources and can be utilized in resistance breeding programs to introduce genetic variability in rapeseed and mustard. These lines had lighter seed colors, resistance to *Alternaria* blight disease and high-temperature tolerance, and they need to be evaluated further with *S. alba*-specific molecular markers. These backcrossed progenies are the first to be reported in the development of trait-specific monosomic alien additional lines. The developed lines will be used to identify the introgressions responsible for *A. brassicae* resistance, high-temperature tolerance, high basal branching and high yield performance.

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Author contributions PK developed the somatic hybrids of *Sinapis alba* + *B. juncea* and the backcross progenies (BC_1, BC_1F_2 and BC_2), collected morphological data and worked on the final copy of the manuscript. KPS conduted the disease screening in BC_1F_2 and BC_2 progenies, analyzed data and drafted and edited manuscript. DB and SK performed the GISH analyses.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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